Chromosome-Specific Painting in *Cucumis* Species Using Bulked Oligonucleotides

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ABSTRACT Chromosome-specific painting is a powerful technique in molecular cytogenetic and genome research. We developed an oligonucleotide (oligo)-based chromosome painting technique in cucumber (*Cucumis sativus*) that will be applicable in any plant species with a sequenced genome. Oligos specific to a single chromosome of cucumber were identified using a newly developed bioinformatic pipeline and then massively synthesized *de novo* in parallel. The synthesized oligos were amplified and labeled with biotin or digoxigenin for use in fluorescence *in situ* hybridization (FISH). We developed three different probes with each containing 23,000–27,000 oligos. These probes spanned 8.3–17 Mb of DNA on targeted cucumber chromosomes and had the densities of 1.5–3.2 oligos per kilobases. These probes produced FISH signals on a single cucumber chromosome and were used to paint homeologous chromosomes in other *Cucumis* species diverged from cucumber for up to 12 million years. The bulked oligo probes allowed us to track a single chromosome in early stages during meiosis. We were able to precisely map the pairing between cucumber chromosome 7 and chromosome 1 of *Cucumis hystrix* in a F_1 hybrid. These two homeologous chromosomes paired in 71% of prophase I cells but only 25% of metaphase I cells, which may provide an explanation of the higher recombination rates compared to the chiasma frequencies between homeologous chromosomes reported in plant hybrids.

KEYWORDS FISH; chromosome painting; oligonucleotides; chromosome pairing

CONSISTENT identification of individual chromosomes in a species is the foundation for successful cytogenetic research. *Drosophila melanogaster* and maize (*Zea mays*) became important genetic model species early in the last century because these species were among the few in which all chromosomes can be identified cytologically (McClintock 1929; Bridges 1935). In the 1970s, chromosome banding techniques became the engine of rapid development of mammalian and clinical cytogenetics. The advent of fluorescence *in situ* hybridization (FISH) in the 1980s provided a common platform that has led to the development of an array of techniques for chromosome identification. Most significantly, DNA sequences from a single chromosome can be isolated and labeled as a "chromosome painting" probe. Chromosome-specific painting has been a powerful tool to detect interchromosomal rearrangements (Speicher *et al.* 1996) and to reveal karyotype evolution among related animal species through cross-species chromosome painting (Ferguson-Smith and Trifonov 2007).

The plant cytogenetics community has devoted significant effort to develop similar techniques for painting individual chromosomes. Unfortunately, FISH experiments using DNA probes prepared from flow-sorted or microdissected plant chromosomes were not successful (Fuchs *et al.* 1996) due to the cross-hybridization of repetitive DNA sequences in the probes that cannot be efficiently blocked. Lysak *et al.* (2001) developed a chromosome-specific painting technique in *Arabidopsis thaliana* by pooling bacterial artificial chromosome (BAC) clones derived from a specific chromosome. The BAC-based probes developed in *A. thaliana* were also used to paint chromosomes in related species, which became a powerful approach to study genome duplication, chromosomal rearrangement, and evolution in Brassicaceae species (Lysak *et al.* 2005, 2006;

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Mandakova and Lysak 2008; Mandakova *et al.* 2010). This technique, however, requires ordered BAC contigs that cover the entire genome of a plant species. More importantly, it relies on the fact that the *A. thaliana* genome is not only very small (125 Mb) (The Arabidopsis Genome Initiative 2000), but also largely euchromatic and that most of the selected BACs contain almost exclusively single- or low-copy sequences. This approach was also applied in another model plant, *Brachypodium distachyon*, and its related species (Idziak *et al.* 2011; Betekhtin *et al.* 2014). Similarly, *B. distachyon* has a relatively small genome (~300 Mb) and ordered BAC contigs covering the entire genome are available. Lou *et al.* (2014) recently demonstrated a strategy of painting individual chromosomes in *Cucumis* species by PCR amplification and pooling of a large number of single-copy sequences (Lou *et al.* 2014). One shortcoming of this approach is the time and cost required to amplify and recover a large number of PCR products to cover an entire chromosome, especially for plant species with large and complex genomes.

Technical advances in DNA synthesis have allowed massively parallel *de novo* synthesis of thousands of independent oligonucleotides (oligos). The massively synthesized oligos have been successfully labeled as FISH probes in mammalian and *Drosophila* species (Boyle *et al.* 2011; Yamada *et al.* 2011; Beliveau *et al.* 2012). This approach has opened a new door to develop chromosome-specific painting probes in plants. Here, we developed a bioinformatic pipeline to select oligos specific to individual chromosomes in plant species. The selected oligos were then synthesized and attached with specific primers at both ends for amplification, *in vitro* transcription, and reverse transcription. The bulked oligos can be labeled using the traditional hapten biotin or digoxigenin and thus the signals from these probes can be amplified using antibodies against biotin and digoxigenin. We developed three different probes with each containing 23,000–27,000 oligos to identify chromosomes of cucumber (*Cucumis sativus*). All three probes generated bright and chromosome-specific FISH signals. The bulked oligo probes were used to successfully paint homeologous chromosomes from several diploid and polyploid *Cucumis* species that diverged from cucumber for up to 12 million years. We also demonstrate that the bulked oligo probes can be used to track homeologous chromosome pairing in early meiotic stages. We were able to examine the chromosome pairing behavior between cucumber chromosome C7 and its homeologous chromosome H1 in a cucumber × *Cucumis hystrix* F_1 hybrid. This technique will be applicable in any plant species with a sequenced genome.

Materials and Methods

Plant materials

Several *Cucumis* species were used in comparative FISH analyses, including *C. sativus* (cv 9930), *C. hystrix* (accession TH1, $2n = 2x = 24$), *Cucumis melo* (cv Top Mark, $2n = 2x = 24$), *Cucumis zeyheri* (PI 315212, $2n = 2x = 24$), *Cucumis subsericeus* (PI 273650, $2n = 4x = 48$), and *Cucumis pustulatus* (PI 343699, $2n = 6x = 72$). To evaluate the potential of cross-species detection of homeologous chromosomes using the bulked oligo probes, two additional species in the family Cucurbitaceae, *Citrullus lanatus* (PI 508443, $2n = 2x = 22$) and *Cucurbita pepo* L. subsp. *pepo* (PI 531323, $2n = 2x = 40$) were also used in FISH. Seeds of all PI lines were obtained from the U.S. National Plant Germplasm System (Ames, IA). An interspecific F_1 hybrid between *C. hystrix* (accession TH1) and cucumber inbred line Gy14 was developed with embryo rescue (Yang *et al.* 2014) and used for homeologous chromosome pairing study.

Bioinformatic pipeline for oligo selection

We developed the Chorus software for designing bulked oligo probes, which are illustrated in Figure 1A. The first step was to eliminate all repetitive sequences in the genome of a target plant species. The repetitive sequences of the cucumber Gy14 genome (Yang *et al.* 2012) (http://cucumber.vcru.wisc.edu/wenglab/home/database.htm) were filtered using RepeatMasker (http://www.repeatmasker.org). The genome sequences were then divided into oligos of 48 nt in a step size of 5 nt. Oligos containing >6 nt of homopolymers were discarded. Each oligo was then aligned to the Gy14 reference genome using BLAT (Kent 2002) to identify those with homologs (>75% similarity over all 48 nt) in the genome. Next, we calculated the temperature (Tm) and hairpin Tm of each oligo using Primer3 (Untergasser *et al.* 2012). Oligos with $d\text{Tm} > 10°$ ($d\text{Tm} = \text{Tm} - \text{hairpin Tm}$) were kept to build a probe database. Probes associated with a specific chromosome or genomic regions were selected from the probe database and visualized along the pseudomolecules using Python and R.

Probe preparation from synthesized oligo libraries

The oligo libraries were synthesized by MYcroarray (Ann Arbor, MI). Each synthesized library contained 400 ng DNA. The libraries were first amplified using an emulsion PCR protocol (Murgha *et al.* 2014). Briefly, the PCR mixture (aqueous phase,100 μl) consisted of ~0.2 ng DNA from the library, 0.5 μM each of F (T7 RNA polymerase promoter plus 6 additional nucleotides) and R (CGTGGTCGCGTCTCA) primers (Figure 1B), 0.2 mM dNTPs, 0.5 μg/μl bovine serum albumin, 4 units of Phusion High-Fidelity polymerase (New England Biolabs, catalog no. M0530S) in 1× high fidelity (HF) buffer. The oil phase (420 μl) contained 4% ABIL EM90 and 0.05% Triton X 100 in mineral oil. The oil phase was continuously stirred at 1000 × *g* at 4°, and the aqueous phase was added. After the emulsion was stirred for an additional 15 min, the reaction was incubated at 98° for 2 min, followed by 30 cycles of 15 sec at 98°, 30 sec at 56°, and 30 sec at 72°, and a final extension at 72° for 5 min. The emulsified PCR mix was successively washed with 1 ml water-saturated diethyl ether and ethyl acetate, and finally with diethyl ether. The remaining diethyl ether was evaporated by incubation at 37° (10–15 min). The reaction was then purified with

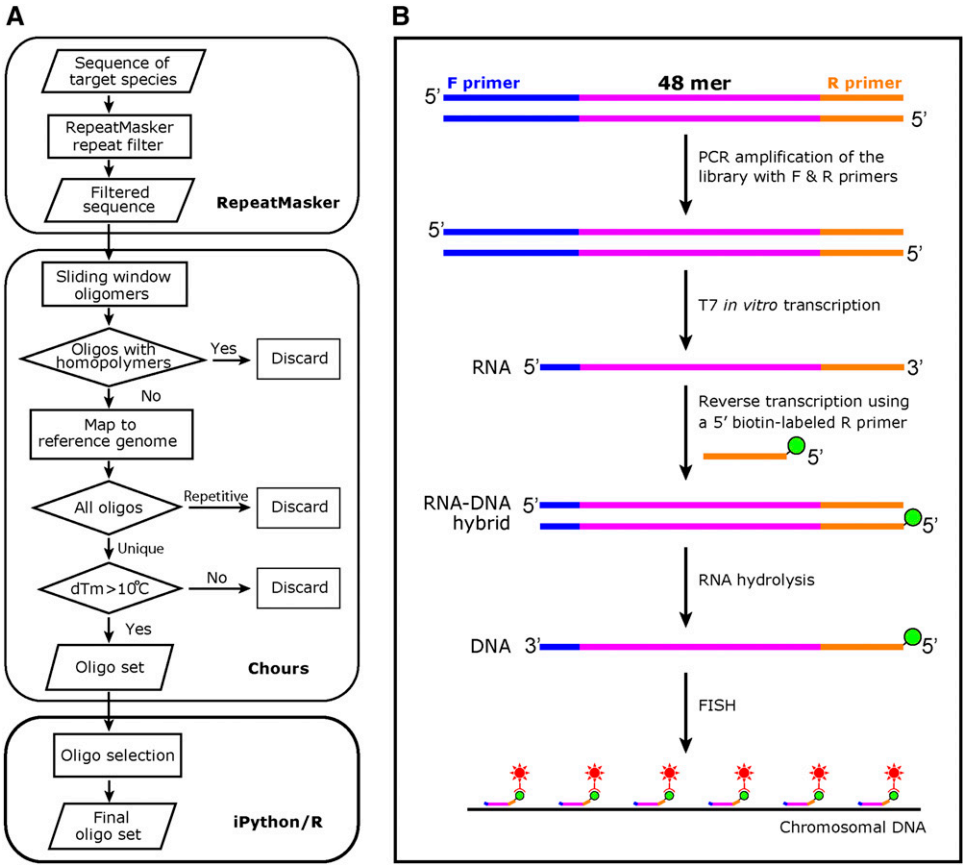

Figure 1 Development of chromosome-specific FISH probes using bulked chromosome-specific oligos. (A) The pipeline of selecting oligos specific to a single chromosome of a plant species with a sequenced genome. (B) Amplification and labeling of bulked oligos for FISH.

a QIAquick PCR purification kit (catalog no. 28104) following the manufacturer's protocol.

Each emulsion PCR resulted in ~1500 ng amplified DNA, and 500 ng product was used for T7 *in vitro* transcription at 37° for 4 hr in a 40-μl reaction with MEGAshortscript T7 Kit (Invitrogen, catalog no. AM1354). The RNA product (~80 μg) was purified by three separate RNeasy spin columns (Qiagen, catalog no. 74104) and eluted with 50 μl of nuclease-free water. Approximately 40 μg RNA was reverse-transcribed using a biotin- or digoxigenin-labeled R primer (5′ biotin-CGTGGTCGCGTCTCA 3′) (2.4 nmol) in 100 μl solution containing 10 mM dithiothreitol, 1.5 mM deoxynucleotide triphosphates, 40 units of SUPERase-In RNase inhibitor (Invitrogen, catalog no. AM2696), and 1000 units of superscript II reverse transcriptase (Invitrogen, catalog no. 18064014). The mixture was incubated at 42° for 4 hr, followed by 15 min at 37° with 2 μl of exonuclease I enzyme to remove unincorporated reverse transcription primers. The reaction (100 μl; RNA:DNA hybrids) was cleaned with the Zymo Quick-RNA MiniPrep kit (catalog no. R1054S). The eluted RNA:DNA hybrids from Zymo-Spin IIICG Column were washed twice with 80 μl of nuclease-free water and then hydrolyzed with 20 units of RNase H (New England Biolabs, catalog no. M0297S) at 37° for 2 hr in a 100-μl solution. Finally, 4 μl RNase A (Thermo Scientific, catalog no. EN0531) was added in the reaction and incubated in a thermocycler programmed as follows: 37° for 1 hr, 70° for 20 min, 50° for 1 hr, 95° for 5 min, ramp down 95°–50° at 0.1°/sec, and 50° for 1 hr. The reaction was cleaned again with Zymo Quick-RNA MiniPrep kit and eluted with 100 μl of nuclease-free water to obtain single-stranded labeled oligos, which had an approximate concentration of 50 ng/μl and can be used for 100 slides. The 400-ng library DNA can be used for a total of 1,200,000 slides.

FISH

The biotin- or digoxigenin-labeled single-stranded oligos prepared from the libraries were directly used as FISH probes. The FISH procedure was essentially the same as regular FISH protocols developed for somatic metaphase and meiotic pachytene chromosomes (Cheng *et al.* 2002). The type III repeat, a satellite repeat located in all cucumber centromeres (Han *et al.* 2008), was used to identify cucumber chromosomes in cucumber × *C. hystrix* F_1 hybrid. Biotin-labeled probes were detected by Alexa Fluor 488 streptavidin; digoxigenin-labeled probes were detected by rhodamine anti-digoxigenin. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole in VectaShield antifade solution (Vector Laboratories, Burlingame, CA). The FISH images were captured using a Hamamatsu CCD camera attached to an Olympus BX51 epifluorescence microscope. The images were processed with Meta Imaging Series 7.5 software. The final contrast of the images was processed using Adobe Photoshop CS3 software.

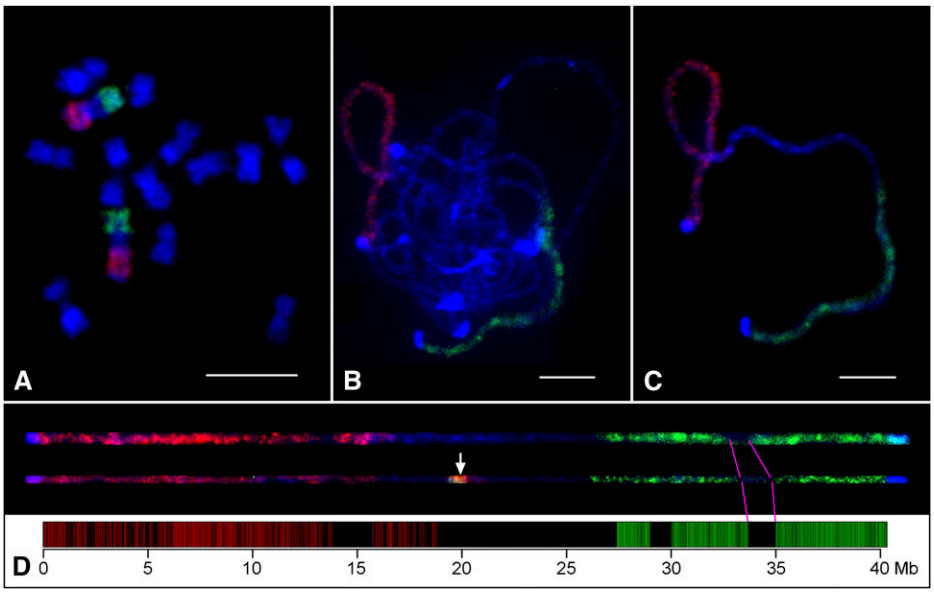

Figure 2 Development of two FISH probes specific to the short and long arms of cucumber chromosome 3. (A) FISH of short-arm-specific probe (red) and long-arm-specific probe (green) of chromosome 3 of cucumber. Note: no cross-hybridization signals were detected on any other chromosomes. (B) FISH of the same two probes to meiotic pachytene chromosomes of cucumber. (C) Cucumber pachytene chromosome 3 was digitally separated from the rest of the chromosomes in B. (D) Locations of 25,000 (red) and 23,000 (green) oligos along the sequence map of cucumber chromosome 3 (bottom). Two pachytene chromosome 3 images were digitally straightened and aligned with the sequence map. A FISH signal gap, marked by purple lines, is visible on both pachytene chromosomes. This gap is likely caused by the lack of oligos within 33.7–35 Mb. The white arrow indicates the FISH signal from the type III repeat associated with cucumber centromeres. Bars, 10 μm.

Results

Development of a bioinformatic pipeline to select chromosome-specific oligos

To develop chromosome-specific FISH probes using bulked oligos, we developed a bioinformatic pipeline to select oligos (see *Materials and Methods*) (Figure 1A). Through this pipeline we were able to identify all nonoverlapping oligos that are unique to a specific chromosome or a chromosomal region of a plant species. Oligos with homology to repetitive DNA sequences or to sequences located on other chromosomes were eliminated. The stringency of oligo selection can be controlled by modifying the parameters of the pipeline, including oligo length, sequence similarity, and melting Tm of the oligos. The "final oligo set" (Figure 1A) was selected based on density (number of oligos per kilobase) and location of oligos for each chromosome or chromosome segment.

To test the bioinformatic pipeline, we designed two probes to cover the majority of the short and long arm, respectively, of cucumber chromosome 3 (Figure 2D). In the cucumber draft genome assembly (Version 1.0 of inbred Gy14) (Yang *et al.* 2012), chromosome 3 spans 40.3 Mb. The short arm probe covered 0–18.82 Mb and included 25,000 oligos. The genomic region between 13.9 and 15.7 Mb is highly repetitive and no oligos were selected from this region (Figure 2D). Overall, the short arm probe had a density of 1.47 oligos per kilobase, excluding the gap region. The long arm probe spanned from 27.4 Mb to the end of the chromosome and contained 23,000 oligos. Two regions on the long arm, 29–30 and 33.7–35 Mb, respectively, are highly repetitive, and no oligos were selected from these regions (Figure 2D); this probe had a density of 2.17 oligos per kilobase, excluding the two gap regions.

Labeling and hybridization of bulked oligos to mitotic and meiotic chromosomes

The oligos were synthesized by the MYcroarray (Ann Arbor, Michigan). Each synthesized oligo contained 48 bp of genomic sequence, a 5′ F primer, which included the T7 RNA polymerase promoter sequence, and a 3′ R primer (Figure 1B). The oligo pool was first amplified by PCR using the F and R primers. The PCR product was then used as a template for T7 *in vitro* transcription. The resulting RNA library was reverse-transcribed using a new R primer that was attached by a biotin or digoxigenin molecule on its 5′ nucleotide (see *Materials and Methods*). RNA hydrolysis was performed on the resulting complementary DNA to produce single-strand DNA molecules. Since each single-strand DNA molecule was attached with biotin or digoxigenin, the pooled molecules were directly used as a FISH probe (Figure 1B).

Both probes prepared from the oligo libraries produced bright FISH signals on cucumber metaphase chromosomes (Figure 2A). As expected, the centromeric region of chromosome 3 was not labeled and was located between the two probes. Although both probes included regions that do not contain any oligos, the FISH signals on the metaphase chromosomes nearly uniformly covered the two arms without unambiguous signal gaps. The probes were also hybridized to meiotic pachytene chromosomes of cucumber (Figure 2, B and C). Nearly uniform FISH signals were detected on the pachytene chromosome. Regions without FISH signals were observed on pachytene chromosome 3. The FISH signal gap likely associated with 33.7–35 Mb on the long arm was consistently detected (Figure 2D). In addition, the intensity

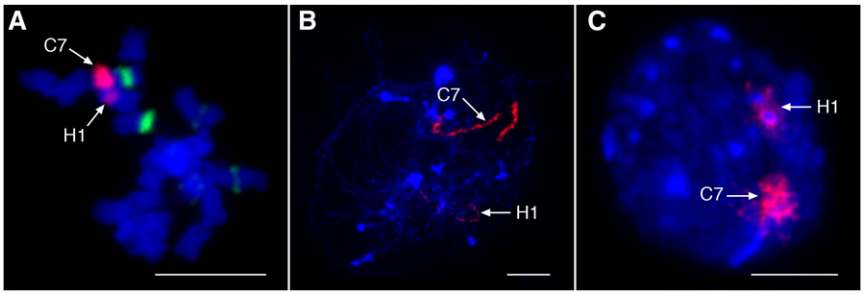

Figure 3 FISH of a chromosome 7-specific probe in cucumber × C. hystrix F_1 hybrid. Arrows point to the signals from cucumber chromosome C7 and *C. hystrix* chromosome H1. (A) FISH on a somatic metaphase cell. Green signals were from the type III repeat-associated cucumber centromeres. (B) FISH on a meiotic pachytene cell. (C) FISH on an interphase nucleus. Bars, 10 μm.

of FISH signals on the pachytene chromosome was not as strong as those on somatic metaphase chromosomes.

Cross-species chromosome painting using bulked oligos

We next investigated the potential of oligo-based probes for cross-species chromosome painting. Cucumber chromosome 7 (C7) has maintained a complete synteny with a single chromosome in the *Cucumis* species without interchromosomal rearrangements (Huang *et al.* 2009; Yang *et al.* 2014). Comparative genetic and genomic studies revealed a high degree of synteny and collinearity in the long arm of C7 (~8 Mb) across the *Cucumis* species and beyond (Yang *et al.* 2014). Thus, C7 provides an ideal target to test cross-species chromosome painting using bulked oligo probes. We developed three different probes to cover the distal 8.3 Mb of C7 (10.91–19.30 Mb in Gy14 assembly). These three probes included 27,000 (3.2/kb), 44,000 (5.2/kb), and 62,000 (7.3/kb) oligos, respectively. All three probes generated high-quality FISH signals on the long arm of C7 (C7L). We did not observe a clear difference of the intensity of the FISH signals derived from these three probes.

We first tested the C7 probe in a hybrid derived from a cross between cucumber and *C. hystrix* ($2n = 2x = 24$), which diverged from *C. sativus* ~4.6 million years ago (MYA) (Sebastian *et al.* 2010). A distinct FISH signal was detected on a single *C. hystrix* chromosome (H1) in both mitotic metaphase and meiotic pachytene cells in the hybrid (Figure 3, A and B). Two separate FISH signal domains were also observed in the interphase nuclei of the hybrid (Figure 3C). However, the signals associated with H1 were consistently weaker than those from C7.

We next performed FISH on metaphase chromosomes from four additional *Cucumis* species, including *C. melo* ($2n = 2x = 24$), *C. zeyheri* ($2n = 2x = 24$), *C. subsericeus* ($2n = 4x = 48$), and *C. pustulatus* ($2n = 6x = 72$). The *Cucumis* genus diverged from other genera in the Cucurbitaceae family ~12 MYA (Sebastian *et al.* 2010). The C7 oligo probe generated FISH signals with similar intensity on specific chromosomes in all four species, with signals on two chromosomes in the two diploid species (Figure 4, C and D), on four chromosomes in tetraploid *C. subsericeus* (Figure 4E), and on six chromosomes in hexaploid *C. pustulatus* (Figure 4F). We also tested the FISH probe on chromosomes of *C. lanatus* (watermelon, $2n = 2x = 22$) and *C. pepo* ($2n = 4x = 40$), which diverged from the cucumber lineage ~20 and ~30 MYA, respectively. We did not detect unambiguous signals in these two species using any of the three probes (Figure 4, G and H).

Pairing of homeologous chromosomes in C. hystrix × cucumber hybrid

Assessment of homeologous chromosome pairing in hybrids between crops and their wild relatives provides important information on the potential of application of the wild germplasm in crop improvement. Chromosome pairing has traditionally been assessed based on analysis of pairing configurations of all chromosomes at metaphase I of meiosis. It has not been possible to analyze the pairing of individual chromosomes in most interspecific hybrids because of the lack of technique to identify individual meiotic chromosomes in most, if not all, plant species. The oligo-based probes provided opportunities to monitor pairing of a specific homeologous chromosome pair throughout meiosis.

The 7C oligo probe from the highly conserved 8.3-Mb region allowed us to track C7 and *C. hystrix* chromosome H1 in prophase I of meiosis in the hybrid (Figure 3B). We analyzed a total of 104 meiotic cells at the zygotene–pachytene stage of meiosis. C7 and H1 were completely separated from each other in 30 (29%) cells (Figure 5A1). The FISH-labeled portions of C7 and H1 partially paired (Figure 5B1) or fully paired (Figure 5C1) in 55 (53%) and 19 (18%) of the cells, respectively. In 4 of the 30 cells in which C7 and H1 did not pair, either C7 or H1 paired with an unidentified chromosome (Figure 5D1).

We then examined the pairing between C7 and H1 at metaphase I (MI) of meiosis. Chromosome pairing configuration at MI was recorded in 169 cells. Only univalents (19 total) were observed in 52 (31%) cells (Figure 6A). The rest of the cells contained one to five bivalents, including one cell containing a trivalent. Of the 117 cells containing at least one bivalent, C7 and H1 remained as univalents in 74 cells (Figure 6B), but paired as a rod bivalent in 42 cells (25% of the 169 total) (Figure 6C). In addition, neither C7 nor H1 paired with any other chromosomes in a bivalent configuration.

Discussion

Bulked oligo probes vs. traditional FISH probes

FISH signals are reliable markers for chromosome identification (Jiang and Gill 2006). Most common FISH probes used in chromosome identification have been repetitive

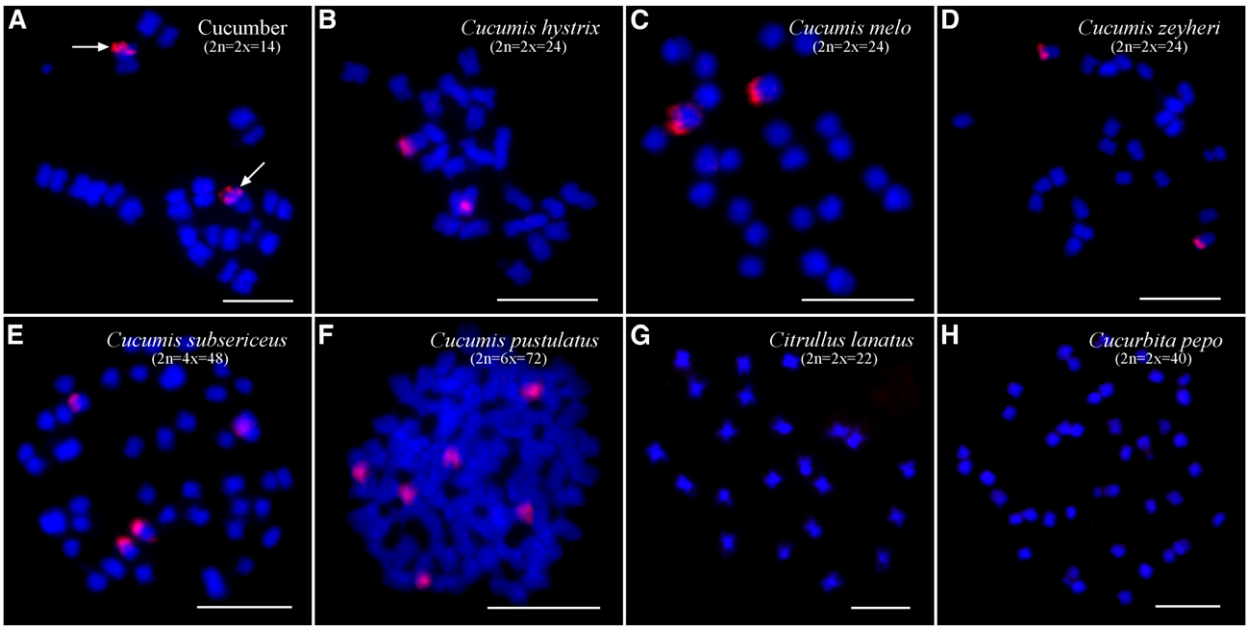

Figure 4 FISH of a cucumber chromosome 7-specific probe (red signals) in cucumber and seven species that have a different genetic distance with cucumber. (A) FISH on metaphase chromosomes of cucumber. Arrows indicate the two copies of C7. (B) FISH on metaphase chromosomes of diploid *C. hystrix*. (C) FISH on metaphase chromosomes of diploid *C. melo*. (D) FISH on metaphase chromosomes of diploid *C. zeyheri*. (E) FISH on metaphase chromosomes of tetraploid *C. subsericeus*. (F) FISH on metaphase chromosomes of hexaploid *C. pustulatus*. (G) FISH on metaphase chromosomes of diploid *C. lanatus*. (H) FISH on metaphase chromosomes of tetraploid *C. pepo*. No FISH signals were observed in G and H. Bars, 10 μm.

DNA elements (Mukai *et al.* 1993; Fransz *et al.* 1998; Lim *et al.* 2000; Kato *et al.* 2004; Fonseca *et al.* 2010; Xiong *et al.* 2011; Chester *et al.* 2012, 2013) or large genomic DNA clones such as BACs (Dong *et al.* 2000; Cheng *et al.* 2001; Kim *et al.* 2002; Wang *et al.* 2008; Findley *et al.* 2010). It often takes a significant effort to develop such probes or probe sets to identify all chromosomes in each species. In addition, repeat-based probes label only specific region(s) of chromosome(s) and may not be useful in identifying the same chromosome(s) of different genotypes or the homeologous chromosomes from different species. Large genomic clones from plant species with large and complex genomes, such as wheat and onion, often contain high proportions of repetitive DNA sequences and cannot be used as chromosome-specific FISH probes (Zhang *et al.* 2004; Janda *et al.* 2006; Suzuki *et al.* 2012).

The bulked oligo probes are superior in resolution and versatility compared to the repetitive sequence- or BAC-based probes on many levels. First, a bulked oligo probe can be designed in various ways to cover an entire chromosome, part of a chromosome, multiple regions of a chromosome, or regions of different chromosomes. Thus, a probe can be specifically designed based on the goals of a research project. Second, a bulked oligo probe can be designed based on sequences conserved among different subspecies or different species. Such probes can be used to identify homeologous chromosomes from different species or subspecies. If sequences are available from two related species, each oligo could be intentionally designed to have equal sequence similarity to both references, thus ensuring a similar FISH signal intensity of the probe in both species. Finally, the bulked oligo probes are cost effective. Although each oligo pool (up to 27,000 oligos) currently costs ~$1500, the cost is expected to drop in the future. In addition, each synthesized library provides enough template DNA for a total of 1,200,000 FISH experiments (see *Materials and Methods*). Therefore, each synthesized library can essentially be used as a permanent resource for the designed probe. In comparison, hundreds or thousands of PCR fragments will be required to paint a single chromosome especially in meiotic cells. Thus, the PCR-based approach (Lou *et al.* 2014) is relatively time-consuming and labor intensive, especially for plant species with a large genome.

We tested probes with densities of 1.5, 2,2, 3.2, 5.2, and 7.3 oligos per kilobase, respectively. These probes generated similar FISH signal intensities on somatic metaphase chromosomes. Thus, a density of 1.5 oligos per kilobase will be sufficient to paint megabase-sized regions on cucumber chromosomes. However, a high density of oligos should be considered for probes to detect small chromosomal regions. Beliveau *et al.* (2012) used a probe with >18 oligos per kilobase to detect a 10-kb region on human chromosomes. We also noted that the probe with 7.3 oligos per kilobase produced slightly stronger signals on cucumber pachytene chromosomes than the probe with 3.2 oligos per kilobase. Therefore, probes with high oligo density are recommended for painting pachytene chromosomes. The biotin- and digoxigenin-labeled probes can be amplified by using additional layers of antibodies. Thus, the signals from probes with relatively low density of oligos can be compensated by using signal amplification systems.

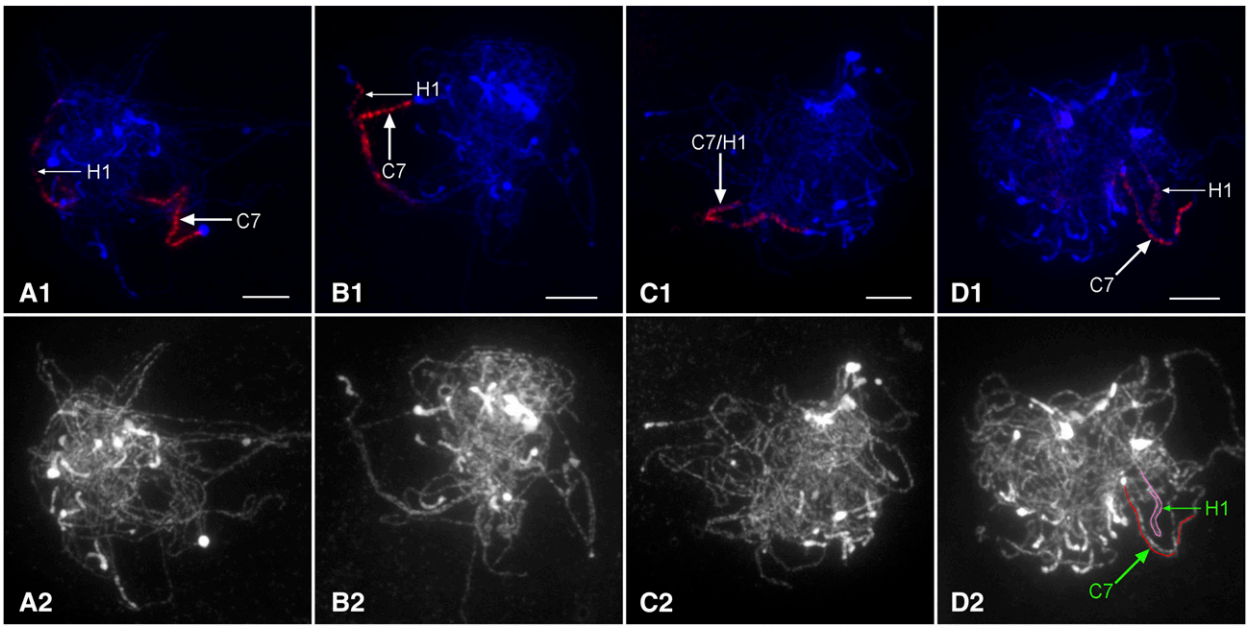

Figure 5 Pairing of between cucumber chromosome C7 and *C. hystrix* chromosome H1 at the zygotene–pachytene stage of meiosis in an F_1 hybrid. All FISH signals (red) were from a bulked oligo probe developed from C7L. (A1) A cell showing that C7 and H1 were not paired. (A2) The original black–white image of the chromosomes in A1. (B1) A cell showing that C7 and H1 were partially paired. The distal ends of the long arms of C7 and H1 were separated. (B2) The original black–white image of the chromosomes in B1. (C1) A cell showing that C7 and H1 were paired completely. (C2) The original black–white image of the chromosomes in C1. (D1) A cell showing that C7 and H1 were not paired. (D2) The original black–white image of the chromosomes in D1. The large green arrow indicates that C7 (red line) paired with an unidentified chromosome in the hybrid. The small green arrow indicates that H1 (pink line) self-paired within the region labeled by FISH signals. Bars, 10 μm.

One limiting factor for developing bulked oligo probes will be the requirement of a reference genome sequence of the target plant species. However, probes may be designed based on sequences from a related species. Our data suggested that the probes developed from cucumber genome sequences can be used in a related species that has been diverged as long as 12 MYA. Alternatively, low-coverage genomic sequences can be produced from the target species and aligned to the reference genome from a related species. Probes can then be designed based on sequences from the target species.

Development of a successful oligo-based FISH probe will rely on identification of the repetitive DNA sequences in the target plant genome. Our current bioinformatic pipeline employed RepeatMasker (http://www.repeatmasker.org) to identify repeats and to filter oligos associated with repetitive sequences (Figure 1). All three cucumber probes generated highly chromosome-specific FISH signals with no visible background signals in nontarget regions (Figure 2A and Figure 3A). However, identification of all repeats in plant species with large and complex genomes will be challenging since the RepeatMasker program may not be able to identify poorly characterized and/or highly degenerated repetitive DNA elements. Recently, several K-mer-based programs have been developed for characterization of repetitive DNA sequences, including Kmasker (Schmutzer *et al.* 2014) and

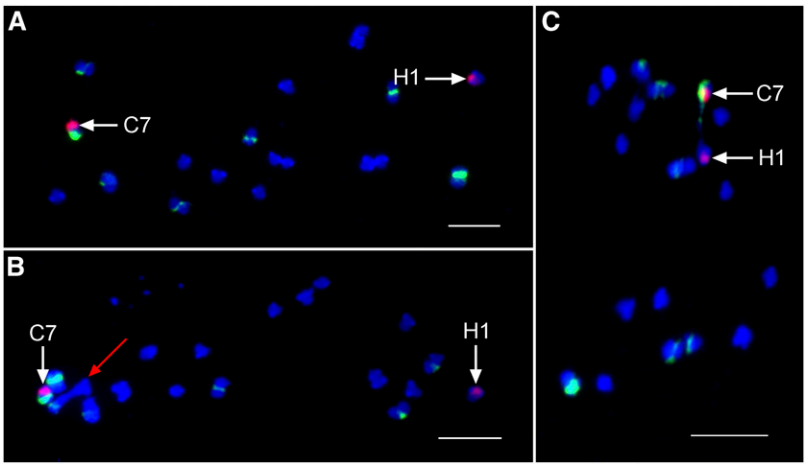

Figure 6 Pairing of cucumber chromosome C7 and *C. hystrix* chromosome H1 at metaphase I of meiosis in an F_1 hybrid. Red FISH signals were from a bulked oligo probe developed from C7L. Green FISH signals were from the type III repeat associated with cucumber centromeres. (A) A metaphase cell showing 19 univalents, including both C7 and H1 (arrows). (B) A metaphase cell showing 17 univalents and one bivalent (red arrow). C7 and H1 (arrows) did not pair in this cell. (C) A metaphase cell showing 17 univalents and one bivalent that consisted of C7 and H1 (arrows). Bars, 10 μm.

RepeatExplorer (Novak *et al.* 2010, 2013). These programs can be used to identify or mask repeats *de novo* without the need of reference databases of known elements and will be especially useful for plant species with poorly sequenced and characterized genomes. Thus, integration of K-mer-based repeat analysis may improve our oligo selection pipeline especially for plants with large and complex genomes.

Mapping homeologous chromosome pairing using bulked oligo probes

Wild relatives of crops often contain valuable traits, such as disease resistances, for crop improvement (Jiang *et al.* 1994; Jansky 2000). These traits can potentially be introgressed into crops by crossing with the wild species and breeding the backcross progenies. The success of wild germplasm introgression will depend on production of a hybrid and recombination between parental chromosomes. The potential level of recombination between parental genomes was often estimated by analyses of chromosomal pairing and chiasma formation at MI in the hybrids. However, the relationship between meiotic recombination and chiasmata has been a debatable subject, especially in plants (Sybenga 1996). Specifically, recombination between homeologous chromosomes in some interspecific hybrids appeared to be higher than the chiasma frequencies estimated by MI chromosome pairing (Sybenga 1996). For example, cultivated rice (*Oryza sativa*, AA genomes) chromosomes rarely paired with chromosomes from wild species *Oryza officinalis* (CC genome) at MI, yet many small interstitial chromosomal segments were transferred from *O. officinalis* into rice (Jena and Khush 1989; Jena *et al.* 1992).

Since recombination events occur in prophase I of meiosis, cytogenetic analysis of chromosomal behavior of interspecific hybrids at early meiotic stages may shed light on the discrepancy between recombination and chiasmata. Although repetitive DNA element-based probes can be used to identify homeologous chromosomes in polyploid species (Mukai *et al.* 1993; Lim *et al.* 2000; Xiong *et al.* 2011; Chester *et al.* 2012), these probes label only specific regions of chromosomes and thus cannot be used to track individual chromosomes at pachytene or earlier meiotic stages because chromosomes are highly extended and often are tangled with each other. We demonstrate that the bulked oligo probes with a density of 1.5–3.2 oligos per kilobase can be used to effectively track chromosomes in early meiosis. Chromosomes C7 and H1 paired as a rod bivalent at MI in 25% of the cells. However, pairing of these two chromosomes was observed in 71% of the zygotene–pachytene cells. C7 and H1 appeared to pair with nonhomologous chromosomes only in ~4% of the zygotene–pachytene cells. Thus, most C7-H1 pairings at the zygotene–pachytene stage were *bona fide* homeologous pairings, and most of these pairings did not result in a chiasma at MI. However, these *bona fide* homeologous pairings may serve as the foundation for the unexplained short exchanged interstitial segments derived from interspecific hybrids (Sybenga 1996). Therefore, it will be important to further investigate the association of these paired homeologous chromosomes with proteins that play roles in meiotic recombination. Antibodies against key proteins in meiotic recombination pathways have been produced in both *A. thaliana* (Osman *et al.* 2011) and rice (Luo *et al.* 2014). The cucumber × *C. hystrix* hybrid provides an ideal material to study the relationship between recombination and homeologous pairing by a combinational approach using FISH with bulked oligos and immunolocalization of meiotic proteins.

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Literature Cited

Beliveau, B. J., E. F. Joyce, N. Apostolopoulos, F. Yilmaz, C. Y. Fonseka *et al.*, 2012 Versatile design and synthesis platform for visualizing genomes with Oligopaint FISH probes. Proc. Natl. Acad. Sci. USA 109: 21301–21306.

Betekhtin, A., G. Jenkins, and R. Hasterok, 2014 Reconstructing the evolution of Brachypodium genomes using comparative chromosome painting. PLoS ONE 9: e115108.

Boyle, S., M. J. Rodesch, H. A. Halvensleben, J. A. Jeddeloh, and W. A. Bickmore, 2011 Fluorescence in situ hybridization with high-complexity repeat-free oligonucleotide probes generated by massively parallel synthesis. Chromosome Res. 19: 901–909.

Bridges, C. B., 1935 Salivary chromosome maps: with a key to the banding of the chromosomes of *Drosophila melanogaster*. J. Hered. 26: 60–64.

Cheng, Z. K., C. R. Buell, R. A. Wing, M. Gu, and J. M. Jiang, 2001 Toward a cytological characterization of the rice genome. Genome Res. 11: 2133–2141.

Cheng, Z. K., C. R. Buell, R. A. Wing, and J. M. Jiang, 2002 Resolution of fluorescence in-situ hybridization mapping on rice mitotic prometaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers. Chromosome Res. 10: 379–387.

Chester, M., J. P. Gallagher, V. V. Symonds, A. V. C. da Silva, E. V. Mavrodiev *et al.*, 2012 Extensive chromosomal variation in a recently formed natural allopolyploid species, Tragopogon miscellus (Asteraceae). Proc. Natl. Acad. Sci. USA 109: 1176–1181.

Chester, M., M. J. Lipman, J. P. Gallagher, P. S. Soltis, and D. E. Soltis, 2013 An assessment of karyotype restructuring in the neoallotetraploid Tragopogon miscellus (Asteraceae). Chromosome Res. 21: 75–85.

Dong, F. G., J. Q. Song, S. K. Naess, J. P. Helgeson, C. Gebhardt *et al.*, 2000 Development and applications of a set of chromosome-specific cytogenetic DNA markers in potato. Theor. Appl. Genet. 101: 1001–1007.

Ferguson-Smith, M. A., and V. Trifonov, 2007 Mammalian karyotype evolution. Nat. Rev. Genet. 8: 950–962.

Findley, S. D., S. Cannon, K. Varala, J. C. Du, J. X. Ma *et al.*, 2010 A fluorescence *in situ* hybridization system for karyotyping soybean. Genetics 185: 727–744.

Fonseca, A., J. Ferreira, T. R. B. dos Santos, M. Mosiolek, E. Bellucci *et al.*, 2010 Cytogenetic map of common bean (*Phaseolus vulgaris* L.). Chromosome Res. 18: 487–502.

Fransz, P., S. Armstrong, C. Alonso-Blanco, T. C. Fischer, R. A. Torres-Ruiz *et al.*, 1998 Cytogenetics for the model system Arabidopsis thaliana. Plant J. 13: 867–876.

Fuchs, J., A. Houben, A. Brandes, and I. Schubert, 1996 Chromosome 'painting' in plants: A feasible technique? Chromosoma 104: 315–320.

Han, Y. H., Z. H. Zhang, J. H. Liu, J. Y. Lu, S. W. Huang *et al.*, 2008 Distribution of the tandem repeat sequences and karyotyping in cucumber (*Cucumis sativus* L.) by fluorescence in situ hybridization. Cytogenet. Genome Res. 122: 80–88.

Huang, S. W., R. Q. Li, Z. H. Zhang, L. Li, X. F. Gu *et al.*, 2009 The genome of the cucumber, *Cucumis sativus* L. Nat. Genet. 41: 1275–1281.

Idziak, D., A. Betekhtin, E. Wolny, K. Lesniewska, J. Wright *et al.*, 2011 Painting the chromosomes of *Brachypodium*: current status and future prospects. Chromosoma 120: 469–479.

Janda, J., J. Safar, M. Kubalakova, J. Bartos, P. Kovarova *et al.*, 2006 Advanced resources for plant genomics: a BAC library specific for the short arm of wheat chromosome 1B. Plant J. 47: 977–986.

Jansky, S., 2000 Breeding for disease resistance in potato. Plant Breed. Rev. 19: 69–155.

Jena, K. K., and G. S. Khush, 1989 Monosomic alien addition lines of rice: production, morphology, cytology, and breeding behavior. Genome 32: 449–455.

Jena, K. K., G. S. Khush, and G. Kochert, 1992 RFLP analysis of rice (*Oryza sativa* L) introgression lines. Theor. Appl. Genet. 84: 608–616.

Jiang, J. M., and B. S. Gill, 2006 Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research. Genome 49: 1057–1068.

Jiang, J. M., B. Friebe, and B. S. Gill, 1994 Recent advances in alien gene transfer in wheat. Euphytica 73: 199–212.

Kato, A., J. C. Lamb, and J. A. Birchler, 2004 Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize. Proc. Natl. Acad. Sci. USA 101: 13554–13559.

Kent, W. J., 2002 BLAT: the BLAST-like alignment tool. Genome Res. 12: 656–664.

Kim, J. S., K. L. Childs, M. N. Islam-Faridi, M. A. Menz, R. R. Klein *et al.*, 2002 Integrated karyotyping of sorghum by in situ hybridization of landed BACs. Genome 45: 402–412.

Lim, K. Y., R. Matyasek, C. P. Lichtenstein, and A. R. Leitch, 2000 Molecular cytogenetic analyses and phylogenetic studies in the *Nicotiana* section Tomentosae. Chromosoma 109: 245–258.

Lou, Q. F., Y. X. Zhang, Y. H. He, J. Li, L. Jia *et al.*, 2014 Single-copy gene-based chromosome painting in cucumber and its application for chromosome rearrangement analysis in *Cucumis*. Plant J. 78: 169–179.

Luo, Q., Y. F. Li, Y. Shen, and Z. K. Cheng, 2014 Ten years of gene discovery for meiotic event control in rice. J. Genet. Genomics 41: 125–137.

Lysak, M. A., P. F. Fransz, H. B. M. Ali, and I. Schubert, 2001 Chromosome painting in *Arabidopsis thaliana*. Plant J. 28: 689–697.

Lysak, M. A., M. A. Koch, A. Pecinka, and I. Schubert, 2005 Chromosome triplication found across the tribe Brassiceae. Genome Res. 15: 516–525.

Lysak, M. A., A. Berr, A. Pecinka, R. Schmidt, K. McBreen *et al.*, 2006 Mechanisms of chromosome number reduction in *Arabidopsis thaliana* and related Brassicaceae species. Proc. Natl. Acad. Sci. USA 103: 5224–5229.

Mandakova, T., and M. A. Lysak, 2008 Chromosomal phylogeny and karyotype evolution in x=7 Crucifer species (Brassicaceae). Plant Cell 20: 2559–2570.

Mandakova, T., S. Joly, M. Krzywinski, K. Mummenhoff, and M. A. Lysak, 2010 Fast diploidization in close mesopolyploid relatives of *Arabidopsis*. Plant Cell 22: 2277–2290.

McClintock, B., 1929 Chromosome morphology in *Zea mays*. Science 69: 629.

Mukai, Y., Y. Nakahara, and M. Yamamoto, 1993 Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence in situ hybridization using total genomic and highly repeated DNA probes. Genome 36: 489–494.

Murgha, Y. E., J. M. Rouillard, and E. Gulari, 2014 Methods for the preparation of large quantities of complex single-stranded oligonucleotide libraries. PLoS ONE 9: e94752.

Novak, P., P. Neumann, and J. Macas, 2010 Graph-based clustering and characterization of repetitive sequences in next-generation sequencing data. BMC Bioinformatics 11: 378.

Novak, P., P. Neumann, J. Pech, J. Steinhaisl, and J. Macas, 2013 RepeatExplorer: a Galaxy-based web server for genome-wide characterization of eukaryotic repetitive elements from next-generation sequence reads. Bioinformatics 29: 792–793.

Osman, K., J. D. Higgins, E. Sanchez-Moran, S. J. Armstrong, and F. C. H. Franklin, 2011 Pathways to meiotic recombination in *Arabidopsis thaliana*. New Phytol. 190: 523–544.

Schmutzer, T., L. Ma, N. Pousarebani, F. Bull, N. Stein *et al.*, 2014 Kmasker: a tool for in silico prediction of single-copy FISH probes for the large-genome species *Hordeum vulgare*. Cytogenet. Genome Res. 142: 66–78.

Sebastian, P., H. Schaefer, I. R. H. Telford, and S. S. Renner, 2010 Cucumber (*Cucumis sativus*) and melon (*C. melo*) have numerous wild relatives in Asia and Australia, and the sister species of melon is from Australia. Proc. Natl. Acad. Sci. USA 107: 14269–14273.

Speicher, M. R., S. G. Ballard, and D. C. Ward, 1996 Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat. Genet. 12: 368–375.

Suzuki, G., Y. Ogaki, N. Hokimoto, L. Xiao, A. Kikuchi-Taura *et al.*, 2012 Random BAC FISH of monocot plants reveals differential distribution of repetitive DNA elements in small and large chromosome species. Plant Cell Rep. 31: 621–628.

Sybenga, J., 1996 Recombination and chiasmata: few but intriguing discrepancies. Genome 39: 473–484.

The Arabidopsis Genome Initiative, 2000 Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B. C. Faircloth *et al.*, 2012 Primer3: new capabilities and interfaces. Nucleic Acids Res. 40: e115.

Wang, K., B. Guan, W. Z. Guo, B. L. Zhou, Y. Hu *et al.*, 2008 Completely distinguishing individual A-genome chromosomes and their karyotyping analysis by multiple bacterial artificial chromosome-fluorescence *in situ* hybridization. Genetics 178: 1117–1122.

Xiong, Z. Y., R. T. Gaeta, and J. C. Pires, 2011 Homoeologous shuffling and chromosome compensation maintain genome balance in resynthesized allopolyploid *Brassica napus*. Proc. Natl. Acad. Sci. USA 108: 7908–7913.

Yamada, N. A., L. S. Rector, P. Tsang, E. Carr, A. Scheffer *et al.*, 2011 Visualization of fine-scale genomic structure by oligonucleotide-based high-resolution FISH. Cytogenet. Genome Res. 132: 248–254.

Yang, L. M., D. H. Koo, Y. H. Li, X. J. Zhang, F. S. Luan *et al.*, 2012 Chromosome rearrangements during domestication of cucumber as revealed by high-density genetic mapping and draft genome assembly. Plant J. 71: 895–906.

Yang, L. M., D. H. Koo, D. W. Li, T. Zhang, J. M. Jiang *et al.*, 2014 Next-generation sequencing, FISH mapping and synteny-based modeling reveal mechanisms of decreasing dysploidy in *Cucumis*. Plant J. 77: 16–30.

Zhang, P., W. L. Li, J. Fellers, B. Friebe, and B. S. Gill, 2004 BAC-FISH in wheat identifies chromosome landmarks consisting of different types of transposable elements. Chromosoma 112: 288–299.

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